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Review

The Role of the Prader-Willi Syndrome Critical Interval for Epigenetic Regulation, Transcription and Phenotype

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Abstract: Prader-Willi Syndrome (PWS) is a neurodevelopmental disorder caused by loss of expression of the paternally inherited genes on chromosome 15q11.2-q13. However, the core features of PWS have been attributed to a critical interval (PWS-cr) within the 15q11.2-q13 imprinted gene cluster, containing the small nucleolar RNA (snoRNA) *SNORD116* and non-coding RNA *IPW* (*Imprinted in Prader-Willi*) exons. *SNORD116* affects the transcription profile of hundreds of genes, possibly via DNA methylation or post-transcriptional modification, although the exact mechanism is not completely clear. *IPW* on the other hand has been shown to specifically modulate histone methylation of a separate imprinted locus, the *DLK1-DIO3* cluster, which itself is associated with several neurodevelopmental disorders with similarities to PWS. Here we review what is currently known of the molecular targets of *SNORD116* and *IPW* and begin to disentangle their roles in contributing to the Prader-Willi Syndrome phenotype.

Keywords: Prader-Willi Syndrome (PWS); epigenetic; transcription; imprinted genes

1. Introduction

Prader-Willi Syndrome (PWS) is a neurodevelopmental disorder characterized by hypotonia and slow growth rate in infancy, followed by severe hyperphagia throughout childhood and adulthood, often leading to obesity and associated problems. Individuals with PWS also exhibit mild to moderate learning disability and, in rare cases, psychosis and other psychiatric conditions [1]. Prader-Willi Syndrome is caused by loss of function mutations affecting the expression of genes on chromosome 15q11.2-q13. The genes in this cluster are imprinted, meaning that they are expressed only from one parental copy of the chromosome, and suppressed on the other. The imprinting of the Prader-Willi gene cluster is established during embryogenesis by a differentially methylated CpG island at the 5′ end of the bicistronic *SNURF-SNRPN* gene [2,3]. This CpG island is known as the imprinting center of the locus [4,5]. On the maternally inherited chromosome, the DNA in the imprinted center is methylated, which leads to the recruitment acetylation and methylation chromatin marks that repress the transcription of maternal PWS genes.

The 15q11.2-q13 region contains some paternally expressed genes, including five protein-coding genes (*MKRN3*, *MAGEL2*, *NECDIN*, *SNURF-SNRPN*), a cluster of small nucleolar RNAs (snoRNAs), and the non-coding RNA *IPW* (Figure 1). The PWS imprinted gene cluster also includes two maternally expressed protein coding genes (*UBE3A* and *ATP10A*) [6]. Loss of expression of paternally inherited genes leads to Prader-Willi syndrome, while the loss of expression of maternally inherted genes, and *UBE3A* in particular, leads to a distinct neurodevelopmental disorder—Angelman syndrome [7].

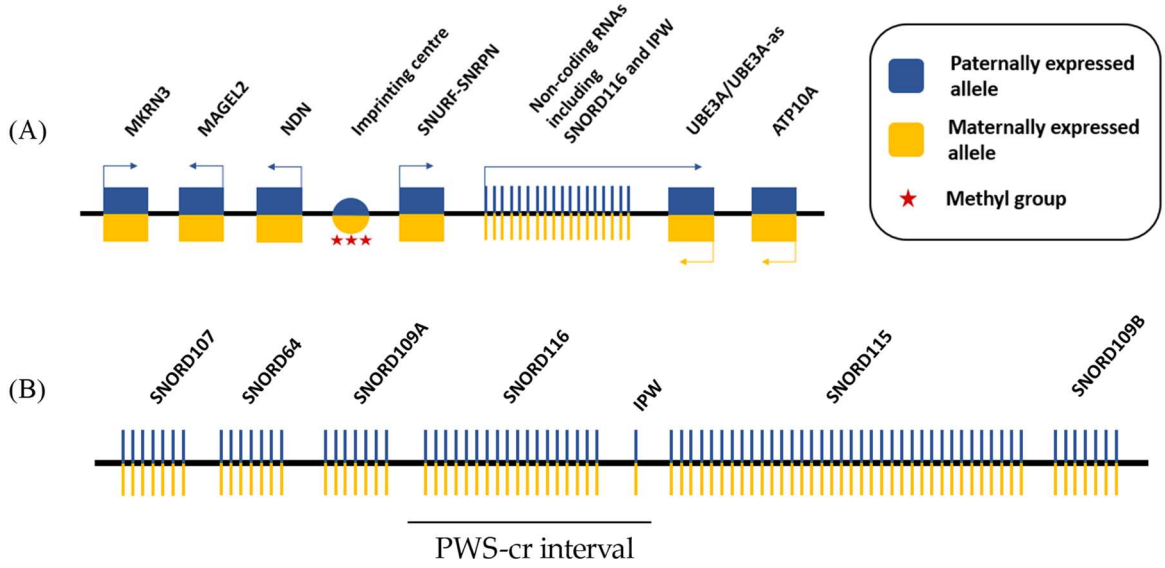

Figure 1. Simplified schematic of the Prader-Willi syndrome locus. (**A**) 15q11.2-q13 imprinted region contains five paternally expressed protein-coding genes and a cluster of paternally expressed non-coding RNAs. The locus also includes two maternally expressed protein-coding genes. The imprinting of the locus is established by DNA methylation of a regulatory region upstream of the paternally expressed *SNURF-SNRPN* allele, which is known as the imprinting center; and (**B**) The non-coding RNAs in the PWS locus contain five clusters of small nucleolar RNAs (snoRNAs) and the Imprinted in Prader Willi (*IPW*) exons. The *SNORD116* cluster together with *IPW* form the PWS-cr interval.

The non-coding RNAs of the PWS cluster play an important role in the Prader-Willi syndrome phenotype by regulating the expression of other genes in a combination of epigenetic and post-transcriptional processes [8–10]. In particular, the Prader-Willi critical region (PWS-cr), containing the *SNORD116* cluster and *IPW*, has been shown to contribute heavily to some of the symptoms of PWS, including the hyperphagia, metabolism, and some anxiety-related behaviors. This review aims to elucidate the current knowledge of the molecular targets of *SNORD116* and *IPW* and how their regulatory role affects the Prader-Willi syndrome phenotype.

2. Functional Consequences of Prader-Willi Syndrome (PWS)-Critical Interval Deletion

The PWS-cr interval is thought to be responsible for the majority of the symptoms associated with PWS, since deletion of the PWS-cr alone has been found sufficient to induce the core PWS phenotype [11]. Due to the high (but not complete) levels of conservation of the PWS cluster between humans and mice, mouse models have become an invaluable tool for studying the physiological and behavioral phenotypes associated with the syndrome. The knock-out of the PWS-cr interval in mice leads to a low birth weight and failure to grow in the first few weeks of life, which is comparable to the phenotype observed in humans [12–15]. Furthermore, some studies on PWS-cr deficient mice have demonstrated a replication of hyperphagia [15,16], although this behavior does not lead to obesity in these models. However, it has to be noted that other studies on this mouse model have shown no differences in the feeding behavior of mutants compared to wild type [17,18], leaving the link between the PWS-cr interval and hyperphagia in contention.

Additionally, loss of gene expression from the PWS-cr interval has been associated with a number of other phenotypes. These include disruption of circadian rhythms both in patients and mouse models, which manifest as an alteration in sleep patterns and specifically REM and Non-REM cycles, as well as a disturbance in metabolism, thermogenesis and food anticipation activity [14,19]. Due to the entanglement of the circadian clock with numerous physiological functions, it is possible

that the disruption of these rhythms is at the base of a multitude of the symptoms associated with Prader-Willi syndrome [20–22].

However, some have questioned whether patients carrying a minimal deletion affecting the PWS-cr present with true PWS [23]. For instance, there is little evidence linking the deletion of the PWS-cr interval with the behavioral and cognitive features characteristic in PWS, although the PWS-cr interval has also been associated with anxiety and some learning disabilities [11,16,24]. Nevertheless, it still remains that deletion of the PWS-cr alone is sufficient to cause the core physiological features of PWS [11] and given this, it is critical that we examine the role of the genes within this interval.

3. *SNORD116*

The *SNORD116* locus contains a cluster of thirty or more snoRNAs, belonging to one of the two repetitive clusters of snoRNAs in the mammalian genome [25]. All of the 15q11.2-q13 PWS region snoRNAs (*SNORD64*, *SNORD107*, *SNORD109*, *SNORD115*, *SNORD116*) are classified as "C/D box snoRNAs". The C and D boxes are short conserved sequence elements, which allow snoRNAs to regulate site-specific methylation of ribosomal RNA (rRNA) [26]. This process is essential for the maintenance of proper ribosomal function, since it is thought to affect the maturity and stability of rRNA. However, none of the C/D box snoRNAs of the PWS region have a direct rRNA target [27], and studies of their functional role have given contradictory results.

Of the snoRNA clusters in the PWS locus, *SNORD115* is the only one that has a clear target. *SNORD115* has been demonstrated to affect the alternative splicing and A-to-I editing of the 2C serotonin receptor pre-RNA [28], which has been associated with hyperphagia and eating abnormalities, as well as with psychiatric disorders [29–31]. The exact mechanisms behind this regulatory effect are unknown. Interestingly, two of the snoRNAs within the *SNORD116* cluster, have been shown to associate with FOX2 from the FOX family, which is a known regulator of splicing [32]. These snoRNAs are left untrimmed from long non-coding RNAs (lncRNAs) found between them, and bind FOX2 in a sno-lncRNA formation [17,32]. However, a recent analysis of hypothalamic transcripts indicated no differences in alternative splicing between PWS-cr deficient mice and wild type [15].

Currently, little is known about the direct molecular target(s) of *SNORD116.* The recognition sites of *SNORD116* are not complementary to any known rRNA, tRNA or snRNA sequence. However, the *SNORD116* cluster has been demonstrated to affect the expression levels of multiple genes. A study by Falaleeva et al. [33] on HEK 293T cells and post-mortem hypothalamus cells of PWS patients demonstrated that *SNORD116* affects the transcription of over 200 genes. This activity was shown to be modified by *SNORD115*, suggesting a synergetic relationship between the two snoRNAs. Falaleeva et al. [33] hypothesize that *SNORD115* and *SNORD116* might form heterodimers, which is compliant with a previously proposed model of C/D box snoRNAs forming dimers [34]. Other RNA-sequencing and microarray studies have also shown that in the absence of the *SNORD116* cluster, the expression of genes that play a role in feeding, skeletal development and circadian rhythm pathways are altered [15,17].

Recently, the *SNORD116* cluster was also shown to potentially affect gene expression through rhythmic DNA methylation [35]. As previously mentioned, the PWS-cr interval has been associated with the regulation of circadian rhythms. This particular role of the interval has been attributed mostly to *SNORD116*, since the snoRNAs of the cluster are predominantly expressed in the neuropeptide Y neurons of the hypothalamus, which is where the "master" circadian molecular clock is located [15,19]. Coulson et al. [35] suggested that the *SNORD116* cluster is involved in the regulation of diurnal rhythm of DNA methylation in the mouse cortex. Using whole genome bisulfite sequencing at six different time points, they demonstrated that over 23,000 CpG sites are methylated rhythmically, and that this rhythm is disrupted in the PWS-cr$^{+/-}$ mice. The dysregulated genes are reportedly enriched for body mass index, cholesterol and diurnal metabolism phenotypes, which are all associated with obesity and could be playing a part in the PWS phenotype.

One of the suggested mechanisms via which the *SNORD116* cluster might regulate rhythmic DNA methylation is through long-noncoding RNAs (lncRNAs) flanking the copies of the *SNORD116* snoRNAs. These lncRNAs are spliced to form an RNA cloud, which associates with transcription factor RBBP5 and regulates the expression of circadian genes *CLOCK*, *CRY1* and *PER2*. This synergetic relationship between the *SNORD116* snoRNAs and their flanking lncRNAs is thought to occur independently of the sno-lncRNA-FOX2 mechanism, which might be involved in assisting alternative splicing [17].

Another possible pathway for *SNORD116* mediated regulation of circadian rhythms is through A-to-I editing of methylation sites. One of the main enzymes behind A-to-I editing known as adenosine-deaminase-acting-on-RNA (ADAR2), is thought to be mediated by C/D box snoRNAs [36], with *SNORD115* being a prime example of one of those snoRNAs [9,28]. ADAR2 was also recently shown to regulate the circadian clocks by performing rhythmic A-to-I editing [37]. Furthermore, elimination of circadian rhythms in mice has been associated with lower weight and less subcutaneous fat [38], which is similar to one of the phenotypes observed in PWS-cr$^{+/-}$ mice. However, there is no body of evidence to support this hypothesis, and although *SNORD116* is now known for playing an essential role in the PWS phenotype, its exact molecular target still remains unclear and requires further studies.

4. Imprinted in Prader-Willi (*IPW*)

All patients carrying a mutation of the *SNORD116* cluster harbor a deletion that spans the non-coding Imprinted in Prader-Willi (*IPW*) gene as well [11]. Similarly, the deletion of *Snord116* in existing mouse models also affects the *Ipw* gene. Consequently, teasing apart the roles played by the *Snord116* cluster and *Ipw* remains a key issue in understanding the neural basis of the PWS phenotype.

The molecular function of *IPW* was unknown until a recent study of a stem cell model of PWS showed that one of the roles of the RNA is to suppress the transcription of another cluster of imprinted genes, namely the *DLK1-DIO3* locus, located on chromosome 14 (Figure 2A) [8]. The proposed mechanism for this regulation suggests that *IPW* is reponsible for the maintenance of repressive chromatin marks at this locus, which lowers expression of the maternally expressed genes (MEGs) in the *DLK1-DIO3* region, and is suggestive of wider imprinted gene network [39]. Specfifically, Stelzer et al. [8] demonstrated that *IPW* interacts with G9A histone methyltransferase, which is capable of catalyzing lysine 9 histone H3 tri-methylation (H3K9me3) (Figure 2B). Pluripotent stem cells from PWS patients (PWS-iPSCs) were shown to exhibit significantly decreased levels of H3K9me3 at a region which controls the expression of the MEGs at the *DLK1-DIO3* cluster. This phenotype is fully rescued by the re-introduction of *IPW* into the PWS-iPSCs [8], which suggests that deletion of *IPW* leads to overexpression of the MEGs in the locus.

Interestingly, very recently Coulson et al. [35] demonstrated that the maternally expressed genes of the *DLK1-DIO3* locus lose diurnal DNA methylation marks in the absence of PWS-cr in mice, and hypothesized that this process is disrupted by the absence of the *SNORD116* cluster. Current evidence of the role of the PWS-cr interval is suggestive of a synergetic relationship between *IPW* and *SNORD116*, which act together to regulate the expression of the *DLK1-DIO3* locus MEGs through histone and DNA methylation, respectively.

Role of the DLK1-DIO3 Locus

Although the phenotypic function of *IPW* has not been explored experimentally, the effects of some of the genes in the *DLK1-DIO3* locus have been studied extensively. The *DLK1-DIO3* imprinted locus contains three paternally expressed protein-coding genes (*DLK1*, *RTL1*, *DIO3*) and multiple maternally expressed non-coding genes, including C/D box snoRNAs and micro-RNAs (miRNAs) (Figure 2A) [40,41]. The maternally expressed miRNAs (miR-379/miR-410) in the *DLK1-DIO3* cluster are of particular importance for the understanding of the PWS phenotype and how it might be affected by *IPW*'s regulatory role. The *DLK1-DIO3* miRNAs are known to target over 400 genes, which are

collectively associated with 67 human diseases, including various physiological anomalies and learning disabilities [42].

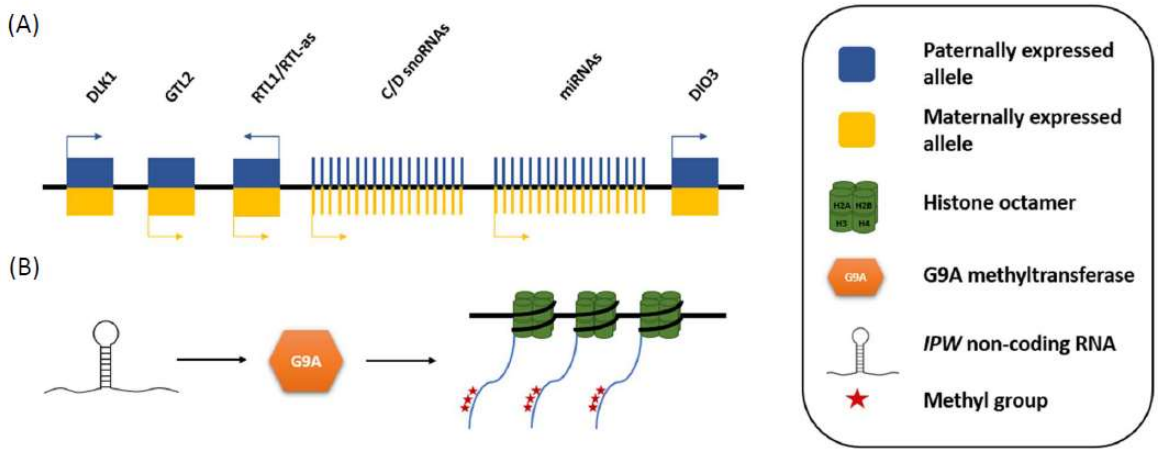

Figure 2. Schematic of how *IPW* down-regulates the expression of maternally expressed genes in the *DLK1-DIO3* locus. (**A**) The *DLK1-DIO3* locus contains three paternally expressed protein coding genes, and multiple maternally expressed non-coding genes (MEGs); and (**B**) *IPW* downregulates the expression of the MEGs by recruiting G9A histone methyltransferase, which adds repressive methyl groups on lysine 9 of the amino acid tail of H3, of the nucleosomes around which the *DLK1-DIO3* MEGs are wrapped.

Deletion of the *miR-379/miR-410* cluster in mouse models leads to increased anxiogenic behavior, as well as with disturbances in metabolic control and homeostasis maintenance [42,43]. These phenotypic outcomes are comparable with the physiological and behavioral phenotypes observed in patients and mouse models carrying a deletion of the PWS-cr interval [8,11,16,24]. Furthermore, overexpression of the MEGs at the *DLK1-DIO3* locus is known to cause Temple syndrome, which is also characterized by symptoms similar to PWS, most notably the early-age hypotonia [44]. The phenotypic overlap between a *DLK1-DIO3* MEGs and the PWS-cr interval suggests that some of the core features of Prader-Willi syndrome may be caused by dysregulation of the maternal genes on the *DLK1-DIO3* locus, rather than directly by (or, more likely, in addition to) the genes in the PWS locus.

Interestingly, the *DLK1-DIO3* and PWS gene clusters encode the only two regions of repetitive snoRNAs in the mammalian genome. Similar to the PWS locus, the *DLK1-DIO3* C/D box snoRNAs also lack well defined rRNA targets and have a very similar genomic organization to the *SNORDs* of the PWS locus [45,46]. These parallels have instigated the hypothesis that perhaps the two imprinted clusters may have been derived from a common ancestor during mammalian evolution [45], which offers another explanation for the similarities in the phenotypes associated with the *DLK1-DIO3* and the PWS-cr interval loci.

5. Conclusions

Although generally caused by loss of paternal gene expression from 15q11-q13, the core of characteristics of Prader-Willi syndrome have been pinpointed to the PWS-cr interval containing the *SNORD116* snoRNA and *IPW* exons. Deletion of the PWS-critical region affects the expression of hundreds of genes via a number of putative mechanisms, and to cause many of the symptoms associated with PWS. Furthermore, recent studies link the PWS-cr interval with the regulation of a distinct imprinted cluster, namely the *DLK1-DIO3* imprinted locus through DNA and histone methylation. Since loss of expression from the maternal genes located in *DLK1-DIO3* is also associated with aspects of the features typical of PWS, these novel discoveries have added a new layer of complexity to our current understanding of the molecular mechanisms behind this syndrome. Teasing apart the potential effects of *SNORD116*, *IPW* and the *DLK1-DIO3* maternally expressed genes would

inform not only our understanding of Prader-Willi syndrome, but also of multiple other pathways related to metabolical, circadian and cognitive health problems.

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Conflicts of Interest: The authors declare no conflict of interest.

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